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AZT-Related Mutation Lys70Arg in Reverse Transcriptase of Human Immunodeficiency Virus Type 1 Confers Decrease in Susceptibility to ddATP in *in Vitro* RT Inhibition Assay

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The genetic basis for didanosine (ddI) resistance in human immunodefeciency virus (HIV-1) has previously been shown to be commonly associated with a Leu to Val change at codon 74 in the HIV-1 RT gene. In this study sequential viral isolates were analyzed from five patients with prior zidovudine (AZT) use who received 6 to 16 months of ddI therapy. Following ddI therapy, viral isolates exhibited an increased AZT susceptibility and decreased ddI susceptibility. Sequence and nested PCR analysis of the HIV-1 RT gene revealed that two viral isolates contained the Leu to Val change at codon 74, and three other isolates with reduced susceptibility to ddI each contained changes at codons 65, 70, and 72. Site-directed mutagenesis was employed to insert specific mutations in RT gene of proviral clone pNL4-3. Analysis of virion-associated reverse transcriptase activity indicated that the Lys70Arg mutation resulted in an enzyme with 2- to 4-fold decreased susceptibility to ddATP. Statistical analysis of the inhibitory concentration for RT activity between pNL4-3 and mutant Lys70Arg viruses obtained in three independent RT inhibition assays was significant ($P = 0.05$) by student t test paired analysis. Drug susceptibility assays on the virus with Lys70Arg mutation showed a marginal decrease in susceptibility to ddI (1.5- to 2-fold) and about 4- to 6-fold decrease in susceptibilty to AZT. Mutations Lys65Glu and Arg72Ser resulted in an impaired RT with greatly diminished functional RT activity. The AZT-associated Lys70Arg mutation results in an RT enzyme with decreased susceptibility to ddATP. © 1996 Academic Press, Inc.

Resistance to Zidovudine (AZT) in human immunodefeciency virus type-1 (HIV-1) is associated with five major mutations in the polymerase domain of reverse transcriptase (RT) [Met41Leu, Asp67Asn, Lys70Arg, Thr215Tyr/Phe, Lys219Gln]. The mutation Lys70Arg alone in the virus confers a 4- to 8-fold decreased susceptibilty to AZT and is the first to occur in patients receiving AZT as monotherapy (*1, 2*). Didanosine (ddI, 2′,3′-dideoxyinosine) and zalacitabine (ddC, 2′,3′-dideoxycytidine) have also been developed to treat HIV-1 infection (*3–5*), and strains of HIV-1 with reduced susceptibilty to ddI have emerged in patients who were switched to ddI after treatment with AZT (*6–8*). Resistance of HIV-1 to ddI has been associated with a single nucleotide change in the RT gene at codon 74 (Leu → Val) (*9*) and the mutations Lys65Arg, Gln151Met, and Met184Val have also conferred decreased susceptibilty to ddI (*10–12*). The decreased susceptibility to ddI due to a stavudine (d4T, deoxy 4′ thymidine)-related mutation Val75Thr has also been reported (*13*) but not confirmed in patients receiving stavudine (*14*).

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The mechanism of the development of ddI resistance conferred by mutations Lys65Arg, Leu74Val, and Met184-Val has been studied using recombinant mutant RTs and the incorporation of dNTPs or ddITP in *in vitro* RT assays (*11, 15–17*). The mutation Thr69Asp in RT is associated with resistance to ddC and no cross-resistance to ddI or AZT is conferred by this mutation (*3*). Cross-resistance between ddI and ddC has been shown with mutations Leu74Val and Met184Val, but cross-resistance between AZT and ddI in clinical isolates using cell culture assays has been infrequently reported (*18–21*). Cross-resistance has not previously been documented in a homogenous population of cloned viruses. The clinical significance of resistance of HIV-1 to AZT is now clearly associated with a poor prognosis (*22*). In this present study, we have analyzed the HIV-1 isolates from five patients who were on AZT treatment for several months and then switched to ddI. By using site-directed mutagenesis and an assay of virion associated RT, we have shown that the mutation Lys70Arg results in an altered HIV-1 RT with decreased susceptibility to ddATP.

Sequential HIV-1 isolates were collected from 42 subjects enrolled in a clinical trial of ddI therapy at Beth Israel Hospital for patients who were intolerant of AZT (ACTG 118). All participants had AIDS, ARC, or asymp-

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TABLE 1

Sensitivity of Clinical HIV-1 Isolates to AZT and ddI and Mutations Observed during Pre- and Post-ddI Therapy

Isolate	Prior AZT therapy (months)	Weeks on ddI	IC_{50} (μM)[a] AZT	ddI	Mutations
HIV-III B	Wild type		0.01	2.20	
Patient 1	9	0	0.22	3.76	Glu^{65}, Gly^{76}, Tyr^{215}
		72	0.05	5.89	Val^{74}, Pro^{116}, Pro^{117}, Phe^{127}, Phe^{135}
Patient 2	6	8	0.19	0.22	Mixture of WT and Tyr^{215}
		72	0.04	6.20	Val^{74}, Tyr^{215}
Patient 3	15	0	0.31	0.41	Arg^{70}, Tyr^{215}
		24	0.04	6.60	Arg^{70} and Ser^{72} or Arg^{70} and Ala^{72}
Patient 4	8	0	0.01	0.70	Arg^{70}
		24	0.07	4.80	Ala102, Arg174
Patient 5	7	0	0.01	0.80	(Ile or Thr or Phe)^{175} [b]
		24	0.17	4.20	Asp^{42} and Arg^{70} or Asp^{42} and Ile^{70})

[a] The IC_{50} values are the average of two independent drug susceptibility assays.

[b] Each of three independent clones analyzed by sequencing showed different amino acid changes at codon 175.

tomatic HIV-1 infection with CD4 $<$ 300 mm^3. Blood was collected from each study participant every 3–6 months for HIV-1 isolation. Phenotypic drug susceptibility to AZT (Burroughs Wellcome Co., Research Triangle Park, NC) and ddI (Calbiochem, La Jolla, CA) were performed employing the ACTG/DoD HIV-1 Drug Susceptibility Assay as described elsewhere (*23*). To detect mutations at RT codon 74 and 215 directly from genomic DNA of pre-ddI and post-ddI therapy samples, a nested primer strategy described elsewhere (*24, 25*) was employed. The presence of mutations in the RT gene derived from clinical isolates was confirmed either by cloning and sequencing the entire RT gene by dideoxynucleotide chain termination (Sequenase v. 2.0 kit, USB) method or by direct PCR amplification using Taq DyeDeoxy Terminator Cycle Sequencing (370A, Applied Biosystem). The observation of the different mutation sites in clinical isolates of 5 patients (Table 1) in this study may be attributed to differential amplification of target DNA molecules, presence of heterogenous population of viruses, and/or selective evolution of resistant viruses in different hosts.

Twenty-one of forty-two subjects terminated the study before 6 months. HIV-1 was isolated from 18/21 subjects who remained on the study drug for at least 6 months. At entry into the study, the mean AZT IC_{50} was 1.03 μM for subjects with $>$6 months of previous AZT therapy and 0.04 μM for subjects with $<$6 months of previous AZT therapy ($P = 0.008$). Increased AZT susceptibility was observed in three post-ddI isolates from patients 1, 2, and 3 (Table 1). The isolate from patient 2 showed the presence of the ddI-related mutation Leu74Val and an AZT-related mutation Thr215Tyr, which explains the decreased susceptibility to AZT for this isolate as reported earlier (*7, 9*). However, the isolates from patients 1 and 3, which also showed decreased susceptibility to AZT after ddI treatment, carried mutations not previously shown to suppress AZT resistance. This suggest that additional factors, outside the RT-encoding region, may contribute to AZT susceptibility (*26*). Previous reports describe an increase in sensitivity to AZT for clinical isolates obtained from patients who were switched to ddI therapy after an initial treatment with AZT (*4, 8, 9, 19, 21, 27*). The mean ddI IC_{50} was 0.98 μM and 1.0 μM for subjects with $<$6 months and $>$6 months of AZT therapy. Among the 18 subjects who remained on the study for at least 6 months, 4 subjects had either a fourfold increase in ddI IC_{50} between the baseline and follow-up samples or a ddI $IC_{50} > 3.0$ μM (Table 1). However, one post-ddI isolate from patient 1 showed negligible decrease in ddI susceptibility even in the presence of Leu74Val mutation (Table 1). On the other hand the isolate from patient 4 showed a sevenfold increase in ddI IC_{50} in the absence of any known ddI-related mutation (Table 1). This indicates the large variability in ACTG/DoD drug susceptibility assay for the measurement of ddI susceptibility in clinical isolates. Only a low level (two- to fourfold) of decrease in susceptibility has been observed with a known ddI-related mutation Leu74Val in cloned viruses (*7, 9*).

An *in vitro* RT inhibition assay was employed to measure directly the effects of specific mutations on the susceptibility of RT to inhibition by ddATP, a metabolically active form of ddI. RT codons 65 to 75 lie in the amino terminal end of HIV-1 RT, a region that corresponds to $\beta3$–$\beta4$ connecting loop in RT-crystal structure (*28*) and mutations Lys65Arg and Leu74Val are associated with decreased susceptibility to ddI and ddATP (*9, 10, 15*). Out of a panel of mutations presented in Table 1, we have selected those which corresponds to $\beta3$–$\beta4$ region of RT for their possible role in conferring decreased susceptibility to ddI. Two of the five viral isolates contained the Leu to Val change at codon 74 known to be associated with ddI resistance. The Lys70Arg mutation has been associatied with an eightfold decrease in suscepti-

TABLE 2

Oligonucleotides Used in This Study

Sr. No.	Mutated RT codon	Mutagenic primers	Nucleotide No.	WT triplet	Mutant triplet
1	Lys70 → Arg	5′TTTTCTCCATCTAGTACTGTC3′	(2768–2748)	AAA	AGA
2	Lys65 → Glu	5′CTGTCTTTTTCCTTTATGGCA3′	(2752–2732)	AAA	GAA
3	Lys65 → Gln	5′CTGTCTTTTTGTTTATGGCA3′	(2752–2732)	AAA	CAA
4	Leu74 → Val	5′GAAATCTACTACTTTTCTCCAT3′	(2780–2759)	TTA	GTA
5	Arg72 → Ser	5′CTACTAATTTGCTCCATTTAGTAC3′	(2775–2752)	AGA	AGC
6	Lys70 → Arg + Arg72 → Ser	5′CTACTAATTTGCTCCATCTAGTATGTC3′	(2775–2748)		
Sequencing primers for RT gene					
		5′TTGCACTTTAAATTTTCCCATT3′	(2535–2555)		
		5′ACAATGGCCATTGACAGAAG3′	(2615–2634)		
		5′AAGTATACTGCATTTACCATACCTAGTATA3′	(2925–2954)		

Note. Highlighted nucleotides were inserted to obtain the mutant codon; Nucleotide numbers in parenthesis are obtained from pNL4-3 sequence Genbank Accession No. M19921 (*42*).

bility to AZT (*1, 2*) and it has not been previously associated with decreased susceptibility to ddI. Three of five patient's pre- and post-ddI isolates showed the presence of the mutation Lys70Arg. We evaluated the effect of Lys70Arg, Lys65Glu, and Arg72Ser mutations on ddI and ddATP sensitivity in cloned viruses.

To insert specific point mutations in RT coding sequences Altered Sites *in vitro* mutagenesis system (Promega, WI) was used according to the directions provided by the manufacturer and others (*29*). Briefly, a 4.3-kb *Sph*I–*Sal*I fragment of proviral clone pNL4-3 was cloned into mutagenesis phagemid pALTER^{TM-1}. Single-stranded DNA from this recombinant phagemid was used to create specific mutations in RT using mutagenic oligonucleotides (Table 2) which differ in one or two nucleotides from the wild-type (WT) sequence. To generate a full-length proviral clone, the 4.3-kb *Sph*I–*Sal*I fragment from pNL4-3 was replaced with the identical fragment of mutagenic vector pALTER-1 carrying various point mutations in RT gene. PHA-stimulated PBMC (5×10^6) were transfected with 5 to 10 μg of plasmid DNA by electroporation. The virus production was monitored by determining the presence of antigen p24 by ELISA kit (Coulter or DuPont) and by assaying the RT-activity by an *in vitro* RT assay which measures the incorporation of [methyl] ^{3}H-TTP into cDNA of a poly(rA) template in the presence of virion associated RT and oligo (dT) (*30*). Upon transfection of PBMC with the full-length clone pNL4-3 and its RT-variants, Lys70Arg and Leu74Val, efficient virus production was seen 5 days after electroporation. In 3 weeks, the p24 antigen concentration rose to 33, 43, and 40 ng/ml in the supernatants collected from pNL4-3, Lys70Arg, and Leu74Val transfectants, respectively. Similarly, the RT-activity was 1.25×10^5, 1.2×10^5, and 1.5×10^5 cpm/ml in a 60-min assay for RT lysates of pNL4-3, Lys70Arg, and Leu74Val viruses, respectively. These results are in agreement with assays employing other proviral clones (e.g., pHXBc2) for mutagenesis studies (*7, 24, 31*). The viral lysates were always tested for the presence of any contaminating DNase or RNase activity prior to RT assay. No such contamination was detected in our RT lysates.

The Lys65Glu change completely interfered with the ability of the virus to replicate, although in other reports Lys65Arg mutant is able to replicate (*32, 33*). Neither the presence of p24 antigen nor any RT-activity was detected in supernatants harvested up to 6 weeks posttransfection. Viral clones carrying the Lys65Gln change instead of Lys65Glu were also made and the pNL4-3 virus carrying mutation Lys65Gln produced only 15 pg/ml of p24 antigen in PBMC after 4 weeks of infection. The Arg72Ser mutation also failed to produce p24 antigen and a construct carrying double mutations (Lys70Arg + Arg72Ser) also did not replicate actively in PBMC. Similarly, the double mutant constructs Lys65Glu + Lys70Arg and Lys65Gln + Lys70Arg were unable to replicate in PBMC. The presence of a heterogenous population of viruses in patient's samples, differential impact of background variations in the HIV RT gene or defective proviruses in patient's circulating PBMC (*34–36*) may be associated with the RT containing these mutations. Additionally, the effect of the variability in the length of the side chains of different amino acids substituted in the RT gene on the production of a functional polymerase cannot be ruled out (*31, 37*).

The mutant clones Lys70Arg and Leu74Val were tested for their ability to incorporate ddATP in a cell-free *in vitro* RT assay using a template-primer combination of homoploymer poly U-oligo (dA) and a substrate [α-^{32}P]dATP as described elsewhere (*38*). Percentage inhibition of RT-activity was calculated after subtracting the background RT-activity in the absence of divalent cation (Mn^{2+}) and the IC_{50} values for WT control and mutant viruses were calculated using statistical program Systat. The *in vitro* RT assay showed a statistically significant difference between WT and mutant RTs (Table 3 and Fig. 1). The average IC_{50} values of three independent asaays were 1.16 $\pm$ 0.04, 3.40 $\pm$ 1.27, and 4.10 $\pm$ 0.28 μM for pNL4-3, Lys70Arg, and Leu74Val viruses, respectively.

TABLE 3

IC_{50} Values for HIV Variant

	IC_{50} (μM)		
	ACTG/DoD drug susceptibility assay		Virion associated RT based assay[a]
HIV variant	AZT	ddI	ddATP
pNL4-3 (WT)	0.13 ± 0.06	4.12 ± 0.65	1.16[b] ± 0.04
Lys70Arg	0.55 ± 0.10	6.85 ± 3.47	3.40[b] ± 1.27
Leu74Val	0.06 ± 0.07	8.14 ± 2.31	4.10[b] ± 0.28

Note. The values given above are the average of multiple independent assays.

[a] Viron associated RT activity was measured in the presence of increasing concentrations of ddATP using template-primer poly U-oligo (dA) and substrate [α-^{32}P]dATP.

[b] Statistical analysis between three pairs of IC_{50} values was significant ($P = 0.05$).

The IC_{50} values were threefold higher for an RT containing Lys70Arg as compared to wild-type pNL4-3 RT enzyme in this assay. Statistical analysis for three pairs of IC_{50} values of pNL4-3 and Lys70Arg viruses was significant ($P = 0.05$) by student *t* test. The RT containing the Leu74Val mutation was included as a positive control for the assay. In an independent assay the IC_{50} values for WT, Lys70Arg, and Leu74Val viral enzymes were 1.2, 2.5, and 3.9 μM, respectively (Fig. 1). The WT recombinant reverse transcriptase obtained from the AIDS Reference Repository, used as a negative control for the assay, was more susceptible to ddATP (IC_{50} 0.06 μM) than virion associated RT of WT pNL4-3 virus (IC_{50} 0.6 μM) in an independent assay (Table 3). The ddI-resistant mutants showed a two- to fourfold decrease in susceptibility to ddI or ddATP in both the cell culture and RT based *in vitro* assays but the RT based assay had less variability and showed a statistically significant difference compared to WT. This differs from AZT-resistant mutants which show a decrease in susceptibility to AZT in cell culture assay but no change by *in vitro* RT assays (*37, 39*).

In the ACTG/DoD consensus PBMC-based drug susceptibility assay the presence of Lys70Arg was associated with a 1.5- to 2.0-fold decrease in ddI susceptibility in comparision to control virus pNL4-3 which was not statistically significant. The positive control virus containing the Leu74Val mutation exhibited a 2.0- to 2.5-fold decrease in ddI susceptibility as compared to WT control pNL4-3. The average ddI IC_{50}s from four independent assays were 4.12 ± 0.65, 6.85 ± 3.47, and 8.14 ± 2.31 μM for WT (pNL4-3), Lys70Arg, and Leu74Val viruses, respectively. Similarly, the mutant virus containing Lys70Arg showed four- to sixfold decrease in susceptibility to AZT as compared to WT control pNL4-3. As shown previously (*9*), the presence of the Leu74Val mutation resulted in a virus exhibiting increased AZT susceptibility in comparision to WT pNL4-3 (Table 3). The ACTG/DoD assay shows approximately threefold variability when assaying ddI resistance and there was considerable overlap in values obtained for WT and mutant viruses. Differences in drug susceptibility of less than threefold are difficult to interpret due to the inherent variability of the ACTG/DoD protocol for ddI.

The mutation Lys70Arg was present in 7 of a total of 25 patient samples from patients who had not received drug therapy (*40*). Upon analysis of quasispecies distributions, extensive heterogenity was observed at codon 70, with genomes harboring either amino acid Lys or Arg. This finding suggests that use of AZT may select for a preexisting variant pool. Our results provide evidence that an AZT-associated mutation Lys70Arg can result in an altered RT with decreased susceptibility to ddATP. It is not possible to predict whether this level of decrease in susceptibility will impact on treatment with ddI. Decreased susceptibility to AZT-resistant virus to dideoxynucleoside agents containing a 3′-azido group has been reported previously using a HeLa cell CD4 plaque assay (*39*).

The mutation Lys70Arg lies in the amino terminal end of the HIV-1 RT, a region that corresponds to the $\beta 3-\beta 4$ connecting loop (*28*) and our results suggest that it may be involved in altered interaction of enzyme with template during cDNA synthesis similar to the mutation Leu74Val (*41*). This study provides direct evidence for the role of the AZT-related mutation Lys70Arg in the development of reduced susceptibility to ddATP in an RT assay. This

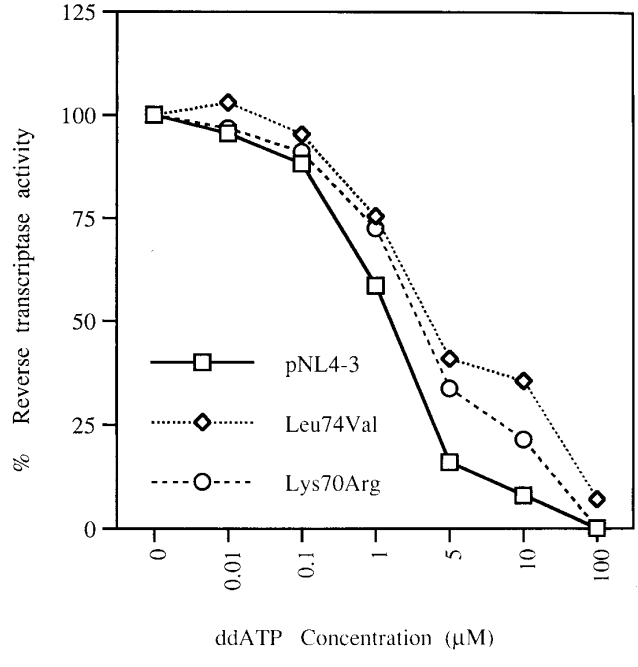

FIG. 1. Inhibition of RT-activity in the presence of ddATP. Virion associated RT lysates were prepared from different HIV variants and the inhibition of RT activity in the presence of increasing concentrations of ddATP was measured. The template-primer used in this assay was poly U-oligo (dA) and the substrate was [α-^{32}P]dATP.

provides direct biochemical evidence that a single amino acid change in this critical region of the RT can result in decreased susceptibilty to nucleosides of diverse structure.

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